

506925
Reed PCT/PTO 07 SEP 2004

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
18 September 2003 (18.09.2003)

PCT

(10) International Publication Number
WO 03/076466 A1

(51) International Patent Classification⁷: **C07K 14/47**

(21) International Application Number: **PCT/KR03/00455**

(22) International Filing Date: 8 March 2003 (08.03.2003)

(25) Filing Language: **Korean**

(26) Publication Language: **English**

(30) Priority Data:
10-2002-0012409 8 March 2002 (08.03.2002) KR
10-2002-0015217 21 March 2002 (21.03.2002) KR
10-2002-0015219 21 March 2002 (21.03.2002) KR

(71) Applicant (*for all designated States except US*): **NEURO-GENEX CO., LTD. [KR/KR]**; Biological Sciences, College of Natural Sciences, Seoul National University, Sillim 9-dong, Gwanak-gu, Seoul 151-747 (KR).

(72) Inventors; and

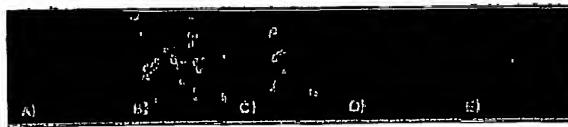
(75) Inventors/Applicants (*for US only*): **SEEN, Dong-Seung**

[KR/KR]; 301-704 Mido Apt., Banpo 4-dong, Seocho-gu, Seoul 137-769 (KR). **PARK, Jae-Yong** [KR/KR]; 201-C, Pyeonghwa Apt., 3-ga, Dangsan-dong, Yeongdeungpo-gu, Seoul 153-043 (KR). **CHOI, Young-Shik** [KR/KR]; 956-4 Hogye 1-dong, Dongan-gu, Anyang, Gyeonggi-do 431-839 (KR). **CHOI, Eun-Wook** [KR/KR]; 910, Dongatown Apt., 1629-2 Bongcheon-dong, Gwanak-gu, Seoul 151-050 (KR). **SON, Ho-Sun** [KR/KR]; 5F, Nakseong-daemyhome, 148-25 Bongcheon 6-dong, Gwanak-gu, Seoul 151-812 (KR). **JUNG, Neon, C.** [KR/KR]; 133-1801 Gwanakdreamtown, Bongcheon 5-dong, Gwanak-gu, Seoul 151-770 (KR). **KANG, Anthony, D.** [US/KR]; 110-1602 Gwanakdreamtown, Bongcheon 5-dong, Gwanak-gu, Seoul 151-770 (KR). **MAENG, Ji-Ung** [KR/KR]; 5-706 Imgwang Apt., Bangbae 3-dong, Seocho-gu, Seoul 137-755 (KR). **SHIN, Jung-Hee** [KR/KR]; 201-33 Myeonmok 8-dong, Jungnang-gu, Seoul 131-811 (KR). **KIM, Kyung-Jin** [KR/KR]; 1102-104 Lucky Apt., Daehyeon-dong, Seodaemun-gu, Seoul 120-757 (KR).

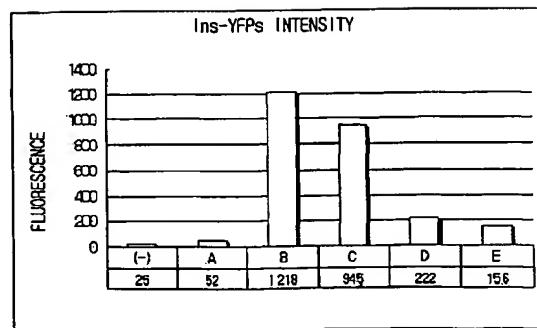
(74) Agent: **NAM, Sang-Sun**; 8th Fl., The Korea Chamber of Commerce & Industry Bldg., 45, Namdaemoonno, 4-ga, Jung-gu, Seoul 100-743 (KR).

[Continued on next page]

(54) Title: ENHANCED INSERTED YELLOW FLUORESCENCE PROTEIN AND ITS APPLICATION



A



B

WO 03/076466 A1

(57) Abstract: Disclosed are mutated genes for green fluorescence proteins and enhanced inserted YFPs expressed therefrom. The mutant proteins not only maintain their fluorescence even at 37 °C, but also exhibit about 20 times stronger fluorescence intensities in comparison to the conventional fluorescence proteins. Accordingly, the mutant fluorescence proteins of the present invention can be used as biosensors for detecting and analyzing the bioactivities of desired materials.

BEST AVAILABLE COPY



- (81) **Designated States (national):** AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) **Designated States (regional):** ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

- 1 -

ENHANCED INSERTED YELLOW FLUORESCENCE PROTEIN AND
ITS APPLICATION

TECHNICAL FIELD

5

The present invention relates to an inserted yellow fluorescence protein for defining functions of any nucleic acid sequences inserted thereto in cells. Furthermore, the present invention relates to biosensors based on the inserted 10 yellow fluorescence proteins for detecting and analyzing bioactivities of any desired materials.

BACKGROUND

15 Green fluorescence protein (hereinafter, referred to as "GFP") was originally isolated from a jellyfish. Since GFP protein consists of a number of 238 amino acids, and since it does not need any other proteins or substrates for fluorescence activation, it has been widely used as a 20 reporter protein. Various types of GFP mutants have been reported. EGFP (enhanced GFP) was prepared by increasing intensity of GFP's fluorescence; and BFP (blue fluorescence protein), CFP (cyan fluorescence protein) and YFP (yellow fluorescence protein) were prepared by modifying fluorescence

- 2 -

spectrum.

In 1998, it was firstly reported that fluorescence intensity of GFP was maintained even after inserting foreign protein or a part of protein or peptides thereto at one or 5 more sites (see NAR 26:623-630). Since then, molecules belonging to the group of the inserted fluorescence proteins, which were developed by R Tsien et al., have been used. YFPins and Camgaroo, which was made by inserting calmodulin to the YFPins, were representative inserted fluorescence 10 proteins designed by R Tsien et al. As for the inserted green fluoresce proteins reported by R Tsien et al., the 145th amino acid sequence of Tyrosine was replaced by "GGTGEL" where restriction enzymes (such as, KpnI and SacI) recognition sites were introduced in order to clone foreign 15 nucleic acid sequences (see PNAS 96:11241-11246). The mutant fluorescence proteins, however, did not show fluorescence activities at 37°C, while they displayed fluorescence activities at 28°C. Thus, they could not be used as biosensors in mammalian cell to measure activities of any 20 desired materials. R Tsien et al. also reported that Camgaroo 2 (Q69M mutant), which was made by substituting 69th amino acid sequence of Glutamine with Methionin, represented fluorescence even at 37°C. However, the fluorescence intensity of the Camgaroo 2 was so weak that it could not be

- 3 -

used in the measurement of calcium at a single cell level. Accordingly, there has been the need to find a novel inserted fluorescence protein having even stronger fluorescence.

Concerning viral disease, since the cause of disease was
5 not defined clearly in general the obtaining of molecules,
which inhibit activities of the disease-causing enzymes, has
been the primary subject for the drug development these days.
In this regard, an efficient cell-based assay system to
monitor activities of target proteins of viral disease was
10 required. Particularly, regarding human Hepatitis C virus,
which cannot be cultivated in a laboratory, the development
of a useful cell-based pharmacological assay system to define
drug efficacy in a cell was greatly required. Although NS3
protease or NS5B RNA polymerase has been considered as an
15 important target protein for the development of drug for the
treatment of viral disease of human Hepatitis C, there has
not been any efficient cell-based assay system to determine
the effects of drug. Until now, recombinant viruses designed
to have life-cycle dependent on NS3 protease activity have
20 been used as cell-based reporting systems for detecting the
activity of NS3 protease. Jang, seung-gi reported in 1996 an
assay system using NS3 protease dependent poliovirus (see
Virology 226:318-26), and Jecyca et al. reported in 1998
other assay system using Sindbis virus for detecting NS3

- 4 -

protease activity (J. Virol 73:561-575). In addition, other assay system using BVDV (bovine viral diarrhea virus) was disclosed in a published document in 2000 (see J. Virol 74: 6339-6347). These assay systems for detecting and analyzing 5 NS3 protease's activity using viruses belonging to Flaviviridae family, which had similar molecular biological features, were proved to have useful features. However, these systems had several problems to be resolved as high throughput systems. These systems regarded to use original 10 viruses as controls, and viral infection problem arose after the cell culture, in the procedures of detecting inhibitors. Therefore, the demand for more efficient and more cost effective assay system is still very high.

It is known that caspase recognizes and cleavages a 15 protein at the site of amino acids following aspartic acid. Caspase-1,4,5,13 cleavage WEHD site, caspase-2,3,7 cleavage DEXD, and caspase-6,8,9,10 cleavage I(/V/L)EXD. In 1998, Xu et al. detected caspase-3 (CPP32) activity using FRET (Fluorescence Resonance Energy Transfer) that was caused by 20 placing DEVD amino acid sequence between GFP and BFP (see NAR 26:2034-2035). Also, BD bioscience clontech designed a system to monitor the activity of caspase-3 through tracing and investigating the YEP within a cell by fusing DEVD-YEP and nuclear export sequence (BD bioscience clontech, PR1Z499W).

- 5 -

However, in detecting caspase activity using FRET, signal/noise (S/N) ratio was too low for practical application in the assay system. In addition, this assay system, which was basically based on protein movement in a 5 cell, required relatively expensive device, and it was difficult to digitize the enzyme activity since the detected results were secondary signals. Accordingly, there have been great needs to find more efficient and cost effective cell-based assay system to detect and analyze the activities of 10 materials.

Thus, in order to provide biosensors for studying activities of numerous desired materials in cells, we carried out researches to develop new type of inserted fluorescence proteins maintaining appropriate fluorescence intensities 15 around 37°C, and thereby we designed enhanced inserted yellow fluorescence proteins by inducing mutations to the inserted fluorescence proteins. Then, we designed a new type of biosensor for detecting activity of NS3 protein inhibitor of human Hepatitis C virus by inserting the NS3 protein 20 inhibitor's substrate recognition sites into the enhanced inserted fluorescence protein. Also, we designed a new type of calcium sensor for determining the amount of calcium in a cell by inserting calmodulin recognition sites into the prepared enhanced inserted fluorescence protein. Furthermore,

- 6 -

we developed a new type of caspase sensor to detect caspase activity in a cell by inserting caspase recognition site into the obtained enhanced inserted fluorescence protein.

5

DETAILED DESCRIPTIONS OF THE INVENTION

Any publications referenced herein are hereby incorporated by reference in this application in order to more fully describe the state of the art to which the present 10 invention pertains.

It is important to understand the present invention to note that all technical and scientific terms used herein, unless otherwise defined, are intended to have the same meaning as commonly understood by one of ordinary skill in 15 the art. The techniques used herein are also well-known to any one with ordinary skill in the art, unless stated otherwise.

Reference to particular buffers, media, reagents, cells, culture conditions and the like, or to some subclass of same, 20 is not intended to be limiting, but should be read to include all such related materials that one of ordinary skill in the art would recognize as being of interest or value in the particular context in which that discussion is presented. For example, it is often possible to substitute one buffer system

- 7 -

or culture medium for another, such that a different but known way is used to achieve the same goals as those to which the use of suggested method, material or composition is directed.

5 It is an object of the present invention to provide an inserted yellow fluorescence protein having enhanced fluorescence intensity at 37°C.

It is another object of the present invention to provide a biosensor to understand various life phenomena in
10 cells.

In order to accomplish these objects, mutations are induced in conventional inserted fluorescence.

Specifically, 145th amino acid of Tyrosine, which is deleted in the conventional inserted fluorescence protein, is
15 introduced. In addition, pcDNA3 (Invitrogen #V79020) vector is prepared, wherein 2 (two) restriction enzyme (BamHI and NheI) recognition sites are placed for efficient cloning of numerous insertion regions. Then, PCR cloning is carried out with the vector to induce mutations. The resultant mutant
20 fluorescence protein maintains intensive fluorescence.

Thus, the obtained and selected mutant inserted yellow fluorescence protein, according to the above method, is characterized by including "YGGSGAS" at 145th amino acid site, wherein the amino acid site is insertion region of foreign

- 8 -

protein or a part of protein. This mutant inserted fluorescence protein is named as Y-Citrine (SEQ.ID No.: 1). The insertion region is designed to function as a binding site that has little electricity in comparison to that of the conventional inserted fluorescence protein. Since the insertion region has restriction enzyme recognition sites, which cannot be found in conventional vectors, it is possible to clone numerous genes into the vector with just one cloning process.

Furthermore, a novel mutant inserted yellow fluorescence protein named as Peridot (SEQ.ID No.: 2), which has additional mutation of replacement of 192nd amino acid of Proline with Lysine (P192L), is provided.

These 2 (two) mutation inserted yellow fluorescence proteins showed about 20 times stronger fluorescence than the conventional inserted fluorescence proteins, under confocal microscopes (see FIG.1).

Furthermore, biosensors for detecting bioactivities of any materials and for monitoring activities of protease are provided with the Peridot. For example, a sensor for assaying NS3 protein inhibitor of human Hepatitis C virus, a calcium sensor and a Caspase sensor are designed.

With regard to the sensor for detecting and analyzing NS3 protein inhibitor of human Hepatitis C virus, it is

- 9 -

provided by inserting amino acid sequences encoding inhibitor's substrate recognition sites into the insertion region of the two developed inserted fluorescence proteins. Specifically, primers of BamHI/5AB-F 5'-GGGGGGATCC GAGGCTGGTG
5 AGGACGTTGT CTGCTGCTCG ATGTC-3' (SEQ. ID No.: 3) and NheI/5AB-R 5'-GGGGCTAGC ACCTGTCCAT GTGTAGGACA TCGAGCAGCA GACAA-3'
(SEQ. ID No.: 4), which encode substrate recognition sites for the NS3 protease, are synthesized and combined, and then are cloned with restriction enzymes. In order to confirm the
10 maintenance and the intensity of fluorescence of the substrate sensor, it is introduced into HeLa cell line and examined and investigated under confocal microscope (see FIG.1). If the substrate biosensor prepared above maintains its fluorescence within a cell, it must be a useful cell-based substrate biosensor for detecting the activity of
15 foreign NS3 protease.

Furthermore, in order for a biosensor binding calcium in a cell to be provided, calmodulin gene, which binds calcium, is introduced into BamHI and/or NheI restriction enzyme
20 recognition sites in Peridot. More specifically, PCR is carried out using calmodulin gene, as a template, and BamHI/CaM F primer 5'-GGGGATCCATGCATGACCAACTGACAGAA-3' (SEQ. ID No.: 5) and NheI/CaM R primer 5'-GGGGCTAGCCTTTGC TGTCATCATTGTAC-3' (SEQ. ID No.: 6). Next, gene cloning is

- 10 -

carried out at BamHI and NheI recognition sites with BamHI and NheI recognition enzymes, and the resulting recombinant gene is named as BCC (bio-cart for calcium). Subsequently, the prepared BCC is transferred to HeLa cell line (ATCC# CCL-
5 2) and is incubated for at least 24 hrs at 37°C. After the incubation, the HeLa cell line having the BCC is examined under confocal microscope to monitor the change of fluorescence while radiating it with Argon laser (480 nm). Specifically, while monitoring the fluorescence, the HeLa
10 cell line is continuously treated with 10 M of cabacol, 1 M of calcium ionopore, 100 mM of calcium solution and calcium-free solution (see FIG.2).

FIG.2A shows photographic images captured at 5-second intervals. FIG.2B illustrates time-dependent graphic images showing changes of calcium for the 4 (four) cells disclosed in FIG.2A. Although each of the 4 (four) cells show different fluorescence intensities dependent on the level of inserted BCC genes, it is observed that all the cells responded in similar ways to external stimuli. It can be clarified by
20 normalizing the fluorescence intensities with the fluorescence intensities detected before imposing the stimuli. FIG.2C shows the normalized graphic images, which strongly support that BCC is an efficient cell-based calcium sensor exactly representing the calcium in cells.

- 11 -

Furthermore, a caspase sensor is provided using Peridot to monitor the activity of caspase in a cell. For this purpose, DEVD amino acid sequence is inserted into the Peridot, and it is named DEVDins. The produced DEVDins is transferred to CHO-K1 (Chinese hamster ovarian) cell line, and then DEVDins expressing cell line is selected and named as CHO-K1-DEVDins. After that, the selected cell line is treated with cell death inducing agent, and the activity of caspase-2/3/7 is detected using quantitative fluorescence image analysis (see FIG. 3). Thus, recombinant fluorescence proteins including caspase recognition amino acid sequences, for example, WEHD for caspase-1/4/5/13, DEXD for caspase-2/3/7 and I(/V/L)EXD for caspase-6/8/9/10, are provided.

15

BRIEF DESCRIPTIONS OF FIGURES

FIG.1A is fluorescence photographs of HeLa cell lines captured by confocal microscope after introducing the inserted fluorescence proteins of the present invention and substrate biosensor for NS3 protease of human Hepatitis C virus. In FIG.1A, A) is for Citrine-Ins, B) is for Y-Citrine, C) is for Peridot, D) is for Y-Citrine-5AB and E) is for Peridot-5AB.

FIG.1B illustrates graphic images representing relative

- 12 -

quantified fluorescence intensities of the inserted fluorescence proteins of the present invention.

FIG.2A is photographs of HeLa cell lines captured at 5-second intervals while continuously treating the cell lines, which include calcium sensor BCC with 10 M of cabacol, 1 M of calcium ionopore, 100 mM of calsuim solution and calcium-free solution.

FIG.2B illustrates quantified graphic images of photographs of FIG.2A under confocal microscope.

FIG.2C illustrates normalized graphs of the graphs of FIG.2B using fluorescence intensities before applying stimulus.

FIG.3A is fluorescence photographs taken after treating HeLa cell lines, which have caspase sensors, with cell death inducing agent of okadaic acid (20 mM/ml).

FIG.3B illustrates quantified graphs of the photographs of FIG.3A.

Preferred embodiments of this invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to those skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and the spirit of the

- 13 -

invention being indicated by the claims that follow the example. The examples herein are meant to exemplify the various aspects of carrying out the invention and not intended to limit the scope of the invention in any way. The 5 examples do not include detailed descriptions of conventional methods employed, such as in the performance of genomic DNA isolation, PCR, and sequencing procedures. Such methods are well known to those skilled in the art and are described in numerous publications. In addition, all the publications 10 referred herein are integrated hereto as references.

EXAMPLES

EXAMPLE 1: DETERMINATION OF FLUORESCENCE INTENSITIES

15 OF INSERTED FLUORESCENCE PROTEINS

In order to obtain a mutant inserted fluorescence protein having enhanced fluorescence intensity at 37°C, 145th amino acid of Tyrosine, which was deleted in the conventional 20 fluorescence protein, was introduced into the conventional inserted yellow fluorescence protein. On the other hand, 2 (two) restriction enzyme recognition sites (HindIII and NotI) were introduced into pcDNA3 vector, which was designed to express genomic materials in mammalian cells, for gene

- 14 -

cloning. For this purpose, 4 (four) primers were used. PCR amplification for 5' region was carried out using YFP (Clontech #6006-1), as a template, and a pair of primers of Hind3/EYFP (Y145MEL)-F 5'-GGGAAAGCTT GGGATGGAGC TCAACAGCCA
5 CAAC-3' primer (SEQ. ID No.: 7) and BamHI,NheI/Yins-R 5'-GTT GCT AGC ACC GGA TCC ACC GTA GTT GTA CTC CAG CTT-3' (SEQ. ID No.: 8). In addition, PCR amplification for 3' region was carried out using YFP (Clontech #6006-1), as a template, and a pair of primers of BamHI, NheI/Yins-F 5'-TAC GGT GGA TCC
10 GGT GCT AGC AAC AGC CAC AAC GTC TAT-3' (SEQ. ID No.: 9) and NotI/EYFP(Y145GGT)-R 5'-GGGGCGGCC GCCTAGGTAC CACCGTTGTA CTC-
3' (SEQ. ID No.: 10). And then, additional PCR amplification was carried out using the above PCR products, as templates, and a pair of primers, Hind3/EYFP(Y145MEL)-F 5'-GGGAAAGCTT
15 GGGATGGAGC TCAACAGCCA CAAC-3' (SEQ. ID No.: 11) and NotI/EYFP(Y145GGT)-R 5'-GGGGCGGCC GCCTAGGTAC CACCGTTGTA CTC-
3' (SEQ. ID No.: 12). Following the application, the resultant mutants were cloned to pcDNA3 vector using Hid3 and
NotI restriction enzymes. The prepared mutants were
20 transferred to HeLa cell lines, and the mutants which maintained the fluorescence at 37°C were selected therefrom. In FIG.1A, A) shows fluoresce photography of Citrine-Ins, which was prepared by R Tsien et al., under the confocal microscope.

- 15 -

Since the sensitivity of confocal microscope is very low, the fluorescence intensity, which can be detected under the general fluorescent microscope, cannot be detected under the confocal microscope. Thus, the photography of FIG.1A had 5 black background. The inserted fluorescence proteins of the present invention, such as B) Y-Citrine and C) Peridot as shown in FIG.1A, represented about 20 times stronger intensities than the conventional fluorescence proteins.

10 EXAMPLE 2: Preparation of a substrate biosensor for NS3 protease of human Hepatitis C virus using inserted fluorescence protein

In order to develop a substrate biosensor for 15 determining the activity of NS3 protease inhibitor, amino acid sequences encoding the NS3 protease's substrate recognition site were introduced into the insertion region of the inserted yellow fluorescence protein. The NS3 protease inhibitor of human Hepatitis C virus recognizes protein-binding sites, such as NS34A, 4A4B, 4B5A and 5A5B, as 20 substrate recognition sites. Particularly, since the 5A5B binding site has excellent feature as substrate, it has been used in protease activity assay system using similar viruses. Therefore, NS5A5B (or 5AB) was used as substrate recognition

- 16 -

site in this example also. In order to insert this substrate recognition site into the protein insertion region of the inserted fluorescence protein, primers of BamHi/5AB-F 5'-GGGGGGATCC GAGGCTGGTG AGGACGTTGT CTGCTGCTCG ATGTC-3' (SEQ. ID No.: 3) and NheI/5AB-R 5'-GGGGGCTAGC ACCTGTCCAT GTGTAGGACA TCGAGCAGCA GACAA-3' (SEQ. ID No.: 4) were synthesized and combined within a tube, and then were subject to cloning with recognition enzymes of BamHI and NheI. Next, the prepared biosensor was transferred to HeLa cell line and examined under the confocal microscope in order to measure fluorescence and its intensity. In FIG.1A, D) shows a fluorescence photography of Y-Citrine-5AB including substrate recognition site within the inserted fluorescence protein Y-Citrine, and E) shows a fluorescence photography of Peridot-5AB including substrate recognition sites within inserted fluorescence protein of Peridot. Although the fluorescence intensities of these sensors were lower than those of the inserted fluorescence proteins not having insertion, they generated significantly enhanced fluorescence intensities in comparison to that of inserted fluorescence protein of R Tsien et al., Citrin-Ins.

Thus, the prepared substrate sensors for NS3 protein inhibitor maintained the fluorescence intensities in cells, and they should be considered as being useful substrate

- 17 -

sensors for detecting NS3 protease activities.

EXAMPLE 3: Preparation of a calcium sensor using inserted fluorescence protein

5 A calcium sensor, which binds calcium in cells, was provided in this example. First, calmodulin gene was transferred to BamHI and NheI recognition sites to prepare the calcium sensor. In order to insert calmodulin into the inserted fluorescence protein, a pair of primers of BamHI/CaM
10 F 5'-GGGGGATCCATGCATGACCAACTGACAGAA-3' (SEQ. ID No.: 5) and NheI/CaM R 5'-GGGGCTAGCCTTGCG TGTTCATCATTGTAC-3' (SEQ. ID No.: 6) were synthesized. Then, PCR was carried out using calmodulin cDNA, as template, and the primers. Subsequently, amplified PCR products were cloned to peridot insertion
15 region with restriction enzymes of BamHI and NheI, and the resultant recombinant gene was named as BCC (Bio-Cart for Calcium) (SEQ. ID No.: 13). Next, the produced BCC was transferred to HeLa cell line, and the fluorescence intensity of BCC within the cell line was examined with Argon Laser
20 (480 nm) under confocal microscope. Considering that the BCC has calmodulin domain at cylinder type loop construct of YFP, it was thought that the structural modification caused by the binding between calmodulin and calcium might result in the change of fluorescence intensity. While determining the

- 18 -

fluorescence intensity of the HeLa cell line including BCC, the cell line was treated with 10 M of cabacol, 1 M of calcium ionopore, 100 mM of calcium solution and calcium-free solution continuously. FIG.2A shows images captured at 5-second intervals, and FIG.2B illustrates graphic images representing time-dependent change of calcium for the 4 (four) cells that were disclosed in FIG.2A. Each of the 4 cells responded to the foreign stimuli in similar ways, although the fluorescence intensities were somewhat different from each other based on the level of insertion of BCC genes. It is clarified in FIG.2C that illustrates graphic images, which are normalized with fluorescence intensities obtained before imposing the stimuli. The enhanced fluorescence intensities, which were induced by the change of calcium amount, were maintained for some time period. Thus, it was confirmed that BCC could be used as a cell-based sensor indicating the amount of calcium in a cell, wherein the amount of calcium changed sensitively to the external stimuli.

Considering the fact that, with the conventional high through-put assay system, it usually takes about 3 minutes to monitor every hole of 96-hole plate, the BCC can be considered as being an excellent biosensor that can be used in various experiments to detect and analyze calcium, since it maintains its fluorescence intensity 5 min or more to the

- 19 -

same external stimuli.

EXAMPLE 4: Preparation of a caspase sensor using inserted fluorescence protein

5

A caspase sensor, which can be used in monitoring the activity of caspase, was prepared in this example using inserted fluorescence protein. DEVD amino acid sequences, which were recognized by caspase 2/3/7, were introduced into 10 insertion region of the inserted fluorescence protein so that the fluorescence intensity of prepared biosensor can be in direct proportion to the change of the activities of caspase. With the prepared biosensor, it was possible to detect activity of caspase 2/3/7 under fluorescent microscope and 15 was possible to digitize the activities. As mentioned in the example 1, a pair of primers of BamHI/DEVD F 5'- GGGGGATCCG CCATCAAGAA TGAAGGAAAG AGAAAAGGCG ACGAGGTG -3' (SEQ. ID No.: 14) and NheI/DEVD R 5'-GGGGCTAGCG GCCACTTCAT CTGTTCCATC CACCTCGTCG CCTTTCTC-3' (SEQ. ID No.: 15) were synthesized 20 and then were combined. Next, the combined primers were cloned into the insertion region in peridot with restriction enzyme, and the resultant product was named DEVDins (SEQ. ID No.: 16). After transferring the DEVDins to CHO-K1 (Chinese hamster ovarian, ATCC #CCL-61) cell line, the cell line was

- 20 -

treated with cell death inducing agent in order to activate caspase to monitor the fluorescence intensity. As a result, it was observed that the fluorescence intensity of the prepared biosensor decreased due to the denaturation of the 5 inserted fluorescence protein by the activated caspase.

INDUSTRIAL APPLICABILITY

The present invention provides novel inserted yellow 10 fluorescence proteins generating about 20 times stronger fluorescence intensity in mammalian cells (at 37°C) in comparison to those of conventional proteins of the same kind. Therefore, with the present enhanced inserted yellow 15 fluorescence proteins, it is possible to carry out cell-based studies for numerous materials.

Insertion of protein-binding site of NS5AB, which is known as a substrate recognition site of NS3 protease of human Hepatitis C virus, provides a substrate biosensor for NS3 protease. Thus, it is possible to develop HCV protease 20 assay system, which can be used in monitoring any desired materials in cells, using the inserted fluorescence proteins, such as Y-Citrine-5AB and Peridot-5AB, as substrates. Accordingly, those skilled in the art can design protease activity assay system by preparing sensor cell line firstly

- 21 -

using the substrate sensor and by introducing NS3 gene of human Hepatitis C virus into the cell line. Furthermore, it is obvious for those skilled in the art to design cell-based activity assay biosensor by inserting substrate recognition site of foreign protein, for example, originating from virus.

Development of a biosensor of the present invention, which can quantify the amount of calcium in cells in real-time, provides essential ways to carry out real-time studies for, such as, cell death, epilepsy and neurosis etc. In addition, with the biosensor, it is possible to develop pharmacological assay system for numerous diseases accompanying the change in the amount of calcium.

Furthermore, the development of caspase sensor of the present invention, which can detect protease activity in cells in real-time, makes it possible to design anti-cancer drug assay system. Likewise, this kind of sensor can be used in numerous disease assay systems by employing various disease-associated proteases.

The substantial feature of the biosensors based on the inserted yellow fluorescence proteins is that these biosensors can be used to detect and to analyze any desired materials in cells in real-time. This unique feature of the biosensors of the present invention provides advantages in deciding suitable time point for assay and in conducting

- 22 -

assays repeatedly, in carrying out pharmacological assays. Furthermore, additional costs for quantification are not required in using these biosensors. Thus, in accordance with the present invention, cost effective high through put 5 pharmacological assay is possible with the enhanced inserted yellow fluorescence proteins.

- 23 -

What is claimed is:

1. An enhanced inserted yellow fluorescence protein, wherein the 145th amino acid of YFP (yellow fluorescence protein) 5 comprises amino acid sequence of YGGSGAS (SEQ.ID No.: 1).
2. A nucleic acid sequence encoding the enhanced inserted yellow fluorescence protein as claimed in claim 1.
- 10 3. The nucleic acid sequence as claimed in claim 2 comprising BamHI and NheI restriction enzyme recognition sites.
4. The enhanced inserted yellow fluorescence protein as claimed in claim 1, wherein the 192nd amino acid of Prolin is 15 replaced by Leucin (SEQ.ID No.: 2).
5. A nucleic acid sequence encoding the enhanced inserted yellow fluorescence protein as claimed in claim 4.
- 20 6. The enhanced inserted yellow fluorescence protein as claimed in claim 1 or claim 4 comprising foreign peptide or protein, or a part of protein.
7. A nucleic acid sequences encoding the enhanced inserted

- 24 -

yellow fluorescence protein as claimed in claim 6.

8. The enhanced inserted yellow fluorescence protein as claimed in claim 6 comprising NS3 protease's substrate
5 recognition site of human Hepatitis C virus (HCV NS3).

9. A nucleic acid sequence encoding the enhanced inserted yellow fluorescence protein as claimed in claim 8.

10 10. The enhanced inserted yellow fluorescence protein as claimed in claim 6, wherein peptide or protein, or a part of protein binds calcium.

11. A nucleic acid sequence encoding the enhanced inserted
15 yellow fluorescence protein as claimed in claim 10.

12. The enhanced inserted yellow fluorescence protein as claimed in claim 6 comprising DEVD amino acid sequence recognized by caspase.

20

13. A nucleic acid sequence encoding the enhanced inserted yellow fluorescence protein as claimed in claim 12.

14. A bioactivity assay system, for use *in vivo* or *in vitro*,

- 25 -

comprising the enhanced inserted yellow fluorescence protein as claimed in claim 6.

15. A biosensor, for use *in vivo* or *in vitro*, comprising the
5 enhanced inserted yellow fluorescence protein as claimed in
claim 1 or claim 4.

1/6

FIG.1A

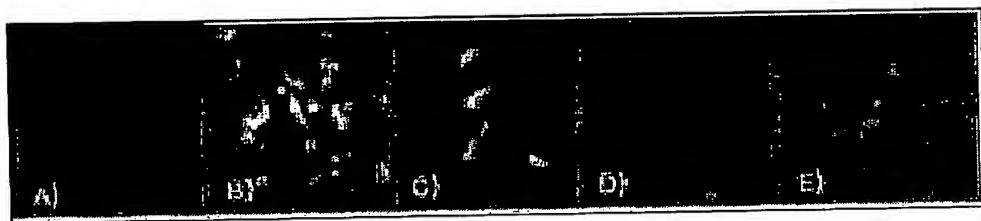
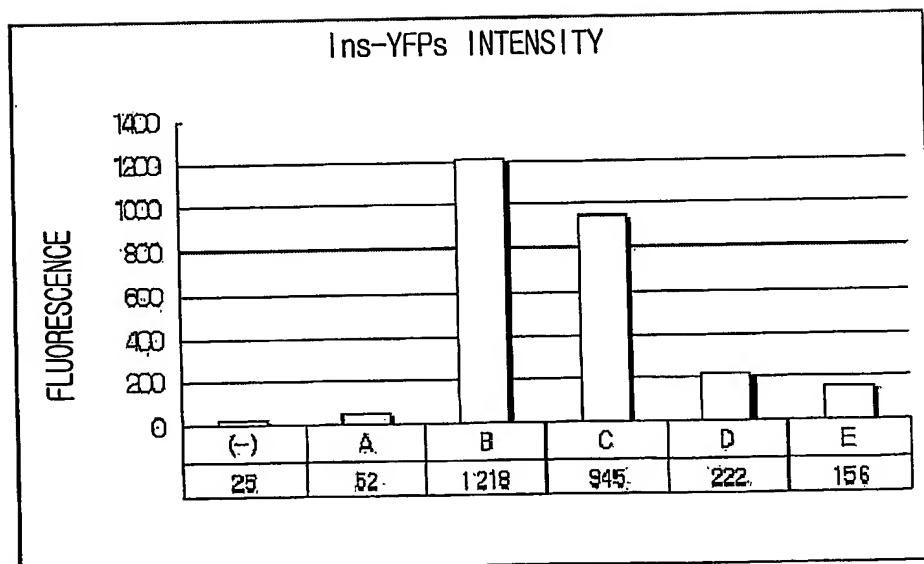
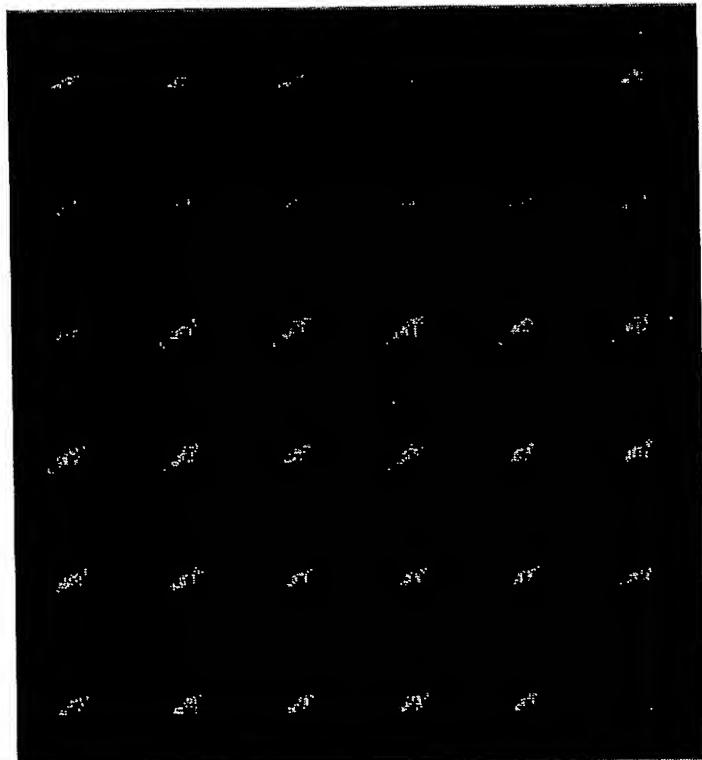
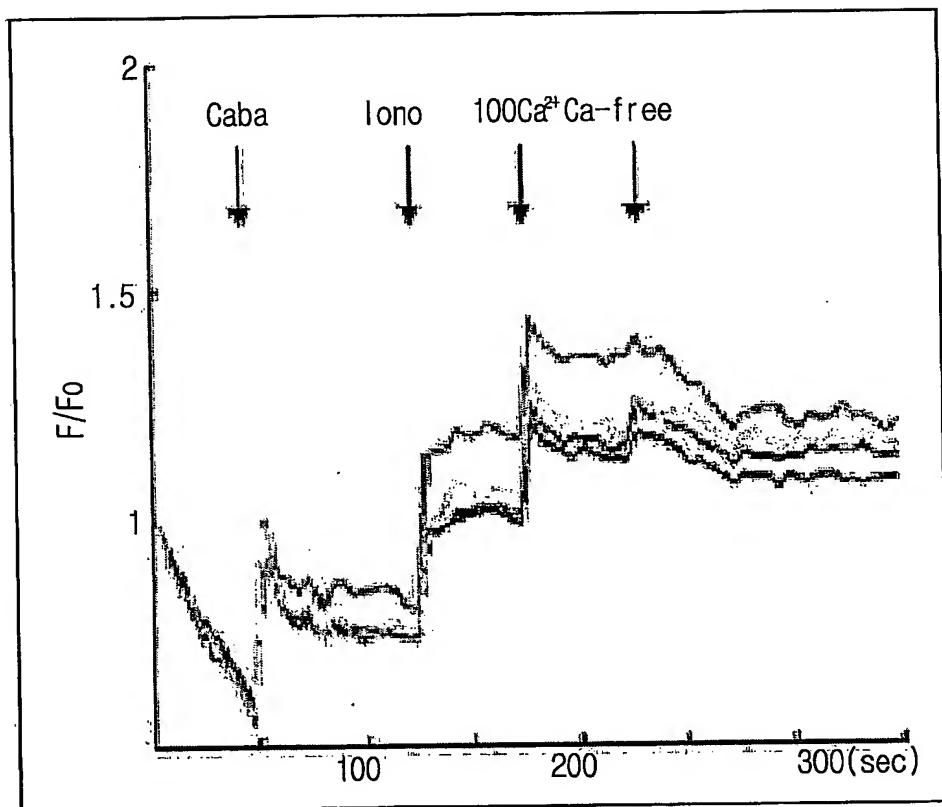


FIG.1B



2/6
FIG.2A

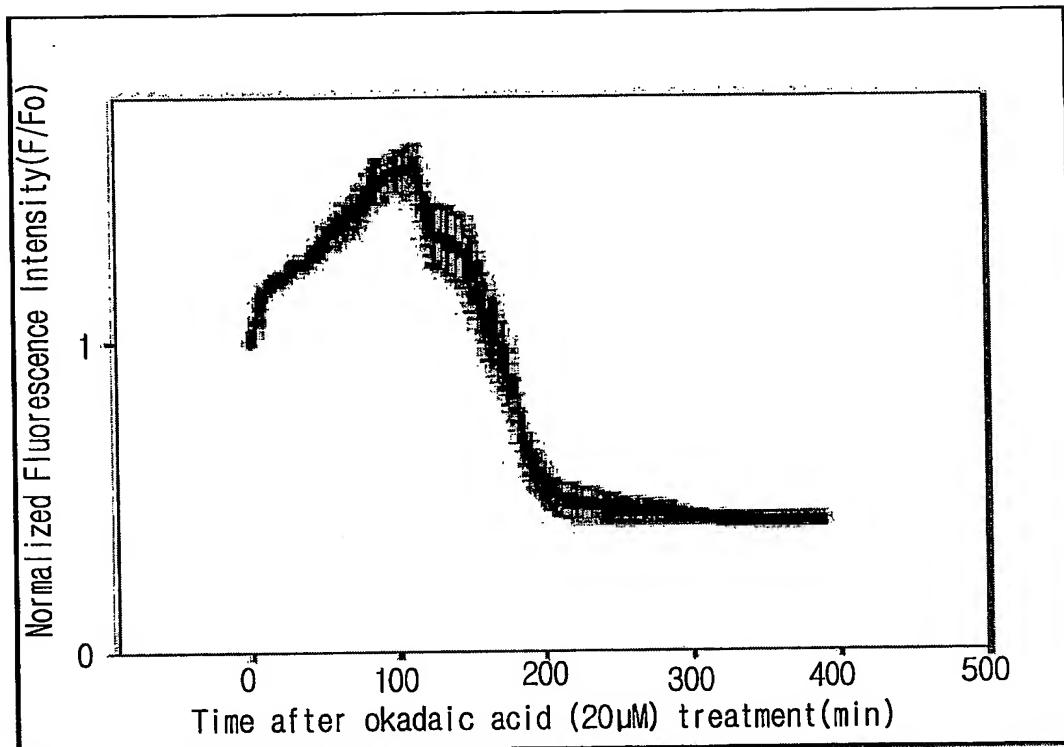
4 / 6
FIG.2C



5/6
FIG.3A

0 min	30 min	60 min	90 min	120 min
150 min	180 min	210 min	240 min	270 min
Time after okadaic acid (20 μ M) treatment(min)				

6/6
FIG.3B



SEQUENCE LIST

<110> Neurogenex Co., Ltd.

<120> ENHANCED INSERTED YELLOW FLUORESCENCE PROTEIN AND ITS APPLICATION

<130> PN0023095.00

<150> KR10-2002-0012409

<151> 2002-03-08

<150> KR10-2002-0015217

<151> 2002-03-21

<150> KR10-2002-0015219

<151> 2002-03-21

<160> 16

<170> KopatentIn 1.71

<210> 1

<211> 738

<212> PRT

<213> Artificial Sequence

<220>

<223> y-citrine of fluorescence protein

<400> 1

Ala Thr Gly Gly Thr Gly Ala Gly Cys Ala Ala Gly Gly Cys Gly

1

5

10

15

Ala Gly Gly Ala Gly Cys Thr Gly Thr Thr Cys Ala Cys Cys Gly Gly
20 25 30

Gly Gly Thr Gly Gly Thr Gly Cys Cys Cys Ala Thr Cys Cys Thr Gly
35 40 45

Gly Thr Cys Gly Ala Gly Cys Thr Gly Gly Ala Cys Gly Cys Gly
50 55 60

Ala Cys Gly Thr Ala Ala Ala Cys Gly Gly Cys Cys Ala Cys Ala Ala
65 70 75 80

Gly Thr Thr Cys Ala Gly Cys Gly Thr Gly Thr Cys Cys Gly Cys
85 90 95

Gly Ala Gly Gly Cys Gly Ala Gly Gly Cys Gly Ala Thr Gly
100 105 110

Cys Cys Ala Cys Cys Thr Ala Cys Gly Gly Cys Ala Ala Gly Cys Thr
115 120 125

Gly Ala Cys Cys Cys Thr Gly Ala Ala Gly Thr Thr Cys Ala Thr Cys
130 135 140

Thr Gly Cys Ala Cys Cys Ala Cys Cys Gly Gly Cys Ala Ala Gly Cys
145 150 155 160

Thr Gly Cys Cys Cys Gly Thr Gly Cys Cys Cys Thr Gly Gly Cys Cys
165 170 175

Cys Ala Cys Cys Cys Thr Cys Gly Thr Gly Ala Cys Thr Ala Cys Cys
180 185 190

Thr Thr Cys Gly Gly Cys Thr Ala Cys Gly Gly Cys Cys Thr Gly Ala
195 200 205

Thr Gly Thr Gly Cys Thr Thr Cys Gly Cys Cys Cys Gly Cys Thr Ala
210 215 220

Cys Cys Cys Cys Gly Ala Cys Cys Ala Cys Ala Thr Gly Ala Ala Gly
225 230 235 240

Cys Ala Gly Cys Ala Cys Gly Ala Cys Thr Thr Cys Thr Thr Cys Ala
245 250 255

Ala Gly Thr Cys Cys Gly Cys Cys Ala Thr Gly Cys Cys Cys Gly Ala
260 265 270

Ala Gly Gly Cys Thr Ala Cys Gly Thr Cys Cys Ala Gly Gly Ala Gly
275 280 285

Cys Gly Cys Ala Cys Cys Ala Thr Cys Thr Thr Cys Thr Thr Cys Ala
290 295 300

Ala Gly Gly Ala Cys Gly Ala Cys Gly Gly Cys Ala Ala Cys Thr Ala
305 310 315 320

Cys Ala Ala Gly Ala Cys Cys Cys Gly Cys Cys Cys Gly Ala Gly
325 330 335

Gly Thr Gly Ala Ala Gly Thr Thr Cys Gly Ala Gly Gly Cys Gly
340 345 350

Ala Cys Ala Cys Cys Cys Thr Gly Gly Thr Gly Ala Ala Cys Cys Gly
355 360 365

Cys Ala Thr Cys Gly Ala Gly Cys Thr Gly Ala Ala Gly Gly Cys
370 375 380

Ala Thr Cys Gly Ala Cys Thr Thr Cys Ala Ala Gly Gly Ala Gly Gly
385 390 395 400

Ala Cys Gly Gly Cys Ala Ala Cys Ala Thr Cys Cys Thr Gly Gly Gly
405 410 415

Gly Cys Ala Cys Ala Ala Gly Cys Thr Gly Gly Ala Gly Thr Ala Cys
420 425 430

Ala Ala Cys Thr Ala Cys Gly Gly Thr Gly Gly Ala Thr Cys Cys Gly
435 440 445

Gly Thr Gly Cys Thr Ala Gly Cys Ala Ala Cys Ala Gly Cys Cys Ala
450 455 460

Cys Ala Ala Cys Gly Thr Cys Thr Ala Thr Cys Ala Thr Gly
465 470 475 480

Gly Cys Cys Gly Ala Cys Ala Ala Gly Cys Ala Gly Ala Ala Gly Ala
485 490 495

Ala Cys Gly Gly Cys Ala Thr Cys Ala Ala Gly Gly Thr Gly Ala Ala
500 505 510

Cys Thr Thr Cys Ala Ala Gly Ala Thr Cys Cys Gly Cys Cys Ala Cys
515 520 525

Ala Ala Cys Ala Thr Cys Gly Ala Gly Gly Ala Cys Gly Gly Cys Ala
530 535 540

Gly Cys Gly Thr Gly Cys Ala Gly Cys Thr Cys Gly Cys Cys Gly Ala
545 550 555 560

Cys Cys Ala Cys Thr Ala Cys Cys Ala Gly Cys Ala Gly Ala Ala Cys
565 570 575

Ala Cys Cys Cys Cys Ala Thr Cys Gly Gly Cys Gly Ala Cys Gly
580 585 590

Gly Cys Cys Cys Cys Gly Thr Gly Cys Thr Gly Cys Cys
595 600 605

Cys Gly Ala Cys Ala Ala Cys Cys Ala Cys Thr Ala Cys Cys Thr Gly
610 615 620

Ala Gly Cys Thr Ala Cys Cys Ala Gly Thr Cys Cys Gly Cys Cys
625 630 635 640

Thr Gly Ala Gly Cys Ala Ala Ala Gly Ala Cys Cys Cys Cys Ala Ala
645 650 655

Cys Gly Ala Gly Ala Ala Gly Cys Gly Cys Gly Ala Thr Cys Ala Cys
660 665 670

Ala Thr Gly Gly Thr Cys Cys Thr Gly Cys Thr Gly Gly Ala Gly Thr
675 680 685

Thr Cys Gly Thr Gly Ala Cys Cys Gly Cys Cys Gly Cys Cys Gly Gly
690 695 700

Gly Ala Thr Cys Ala Cys Thr Cys Thr Gly Gly Cys Ala Thr Gly
705 710 715 720

Gly Ala Cys Gly Ala Gly Cys Thr Gly Thr Ala Cys Ala Ala Gly Thr
725 730 735

Ala Ala

<210> 2

<211> 738

<212> PRT

<213> Artificial Sequence

<220>

<223> Peridot of fluorescence protein

<400> 2

Ala Thr Gly Gly Thr Gly Ala Gly Cys Ala Ala Gly Gly Cys Gly
1 5 10 15

Ala Gly Gly Ala Gly Cys Thr Gly Thr Thr Cys Ala Cys Cys Gly Gly
20 25 30

Gly Gly Thr Gly Gly Thr Gly Cys Cys Cys Ala Thr Cys Cys Thr Gly
35 40 45

Gly Thr Cys Gly Ala Gly Cys Thr Gly Gly Ala Cys Gly Gly Cys Gly
50 55 60

Ala Cys Gly Thr Ala Ala Ala Cys Gly Gly Cys Cys Ala Cys Ala Ala
65 70 75 80

Gly Thr Thr Cys Ala Gly Cys Gly Thr Gly Thr Cys Cys Gly Gly Cys
85 90 95

Gly Ala Gly Gly Gly Cys Gly Ala Gly Gly Cys Gly Ala Thr Gly
100 105 110

Cys Cys Ala Cys Cys Thr Ala Cys Gly Gly Cys Ala Ala Gly Cys Thr
115 120 125

Gly Ala Cys Cys Cys Thr Gly Ala Ala Gly Thr Thr Cys Ala Thr Cys
130 135 140

Thr Gly Cys Ala Cys Cys Ala Cys Cys Gly Gly Cys Ala Ala Gly Cys
145 150 155 160

Thr Gly Cys Cys Cys Gly Thr Gly Cys Cys Cys Thr Gly Gly Cys Cys

165

170

175

Cys Ala Cys Cys Cys Thr Cys Gly Thr Gly Ala Cys Thr Ala Cys Cys

180

185

190

Thr Thr Cys Gly Gly Cys Thr Ala Cys Gly Gly Cys Cys Thr Gly Ala

195

200

205

Thr Gly Thr Gly Cys Thr Thr Cys Gly Cys Cys Cys Gly Cys Thr Ala

210

215

220

Cys Cys Cys Cys Gly Ala Cys Cys Ala Cys Ala Thr Gly Ala Ala Gly

225

230

235

240

Cys Ala Gly Cys Ala Cys Gly Ala Cys Thr Thr Cys Thr Thr Cys Ala

245

250

255

Ala Gly Thr Cys Cys Gly Cys Cys Ala Thr Gly Cys Cys Cys Gly Ala

260

265

270

Ala Gly Gly Cys Thr Ala Cys Gly Thr Cys Cys Ala Gly Gly Ala Gly

275

280

285

Cys Gly Cys Ala Cys Cys Ala Thr Cys Thr Thr Cys Thr Thr Cys Ala

290

295

300

Ala Gly Gly Ala Cys Gly Ala Cys Gly Gly Cys Ala Ala Cys Thr Ala

305

310

315

320

Cys Ala Ala Gly Ala Cys Cys Cys Gly Cys Gly Cys Cys Gly Ala Gly

325

330

335

Gly Thr Gly Ala Ala Gly Thr Thr Cys Gly Ala Gly Gly Cys Gly

340

345

350

Ala Cys Ala Cys Cys Cys Thr Gly Gly Thr Gly Ala Ala Cys Cys Gly

355 360 365

Cys Ala Thr Cys Gly Ala Gly Cys Thr Gly Ala Ala Gly Gly Cys

370 375 380

Ala Thr Cys Gly Ala Cys Thr Thr Cys Ala Ala Gly Gly Ala Gly Gly

385 390 395 400

Ala Cys Gly Gly Cys Ala Ala Cys Ala Thr Cys Cys Thr Gly Gly Gly

405 410 415

Gly Cys Ala Cys Ala Ala Gly Cys Thr Gly Gly Ala Gly Thr Ala Cys

420 425 430

Ala Ala Cys Thr Ala Cys Gly Gly Thr Gly Gly Ala Thr Cys Cys Gly

435 440 445

Gly Thr Gly Cys Thr Ala Gly Cys Ala Ala Cys Ala Gly Cys Cys Ala

450 455 460

Cys Ala Ala Cys Gly Thr Cys Thr Ala Thr Ala Thr Cys Ala Thr Gly

465 470 475 480

Gly Cys Cys Gly Ala Cys Ala Ala Gly Cys Ala Gly Ala Ala Gly Ala

485 490 495

Ala Cys Gly Gly Cys Ala Thr Cys Ala Ala Gly Gly Thr Gly Ala Ala

500 505 510

Cys Thr Thr Cys Ala Ala Gly Ala Thr Cys Cys Gly Cys Cys Ala Cys

515 520 525

Ala Ala Cys Ala Thr Cys Gly Ala Gly Gly Ala Cys Gly Gly Cys Ala

530 535 540

Gly Cys Gly Thr Gly Cys Ala Gly Cys Thr Cys Gly Cys Cys Gly Ala

545 550 555 560
Cys Cys Ala Cys Thr Ala Cys Cys Ala Gly Cys Ala Gly Ala Ala Cys
565 570 575
Ala Cys Cys Cys Cys Ala Thr Cys Gly Gly Cys Gly Ala Cys Gly
580 585 590
Gly Cys Cys Thr Cys Gly Thr Gly Cys Thr Gly Cys Thr Gly Cys Cys
595 600 605
Cys Gly Ala Cys Ala Ala Cys Cys Ala Cys Thr Ala Cys Cys Thr Gly
610 615 620
Ala Gly Cys Thr Ala Cys Cys Ala Gly Thr Cys Cys Gly Cys Cys Cys
625 630 635 640
Thr Gly Ala Gly Cys Ala Ala Ala Gly Ala Cys Cys Cys Cys Ala Ala
645 650 655
Cys Gly Ala Gly Ala Ala Gly Cys Gly Cys Gly Ala Thr Cys Ala Cys
660 665 670
Ala Thr Gly Gly Thr Cys Cys Thr Gly Cys Thr Gly Gly Ala Gly Thr
675 680 685
Thr Cys Gly Thr Gly Ala Cys Cys Gly Cys Cys Gly Cys Cys Gly Gly
690 695 700
Gly Ala Thr Cys Ala Cys Thr Ala Thr Cys Gly Gly Cys Ala Thr Gly
705 710 715 720
Gly Ala Cys Gly Ala Gly Cys Thr Gly Thr Ala Cys Ala Ala Gly Thr
725 730 735
Ala Ala

<210> 3
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> BamHi/5AB-F primer

<400> 3
ggggggatcc gaggctggtg aggacgttgt ctgcgtcgatgc

45

<210> 4
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Nhe1/5AB-R primer

<400> 4
gggggcttagc acctgtccat gtgttaggaca tcgagcagca gacaa

45

<210> 5
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> BamHI/CaM F primer

<400> 5

ggggatcca tgcatgacca actgacagaa

30

<210> 6

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> NheI/CaM R primer

<400> 6

ggggctagcc ttgtgtca tcattttac

30

<210> 7

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Hind3/EYFP(Y145MEL)-F primer

<400> 7

gggaagctt gggatggagc tcaacagcca caac

34

<210> 8

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> BamHI,Nhe1/Yins-R primer

<400> 8

gttgctagca ccggatccac cgtagttgta ctccagctt

39

<210> 9

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> BamHI,Nhe1/Yins-F primer

<400> 9

tacggtgttac ccgggttag caacagccac aacgtctat

39

<210> 10

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> NotI/EYFP(Y145GGT)-R primer

<400> 10

gggggcggcc gcctaggtac caccgttgta ctc

33

<210> 11

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Hind3/EYFP(Y145MEL)-F primer

<400> 11

gggaaagctt gggatggagg tcaacagccca caac

34

<210> 12

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> NotI/EYFP(Y145GGT)-R primer

<400> 12

gggggcggcc gccttaggtac caccgttgta ctc

33

<210> 13

<211> 1182

<212> DNA

<213> Artificial Sequence

<220>

<223> Bio-Cart for Calcium

<400> 13

atggtgagca agggcgagga gctgttcacc ggggtggtgc ccatcctggt cgagctggac

60

ggcgacgtaa acggccacaa gttcagcgtg tccggcgagg gcgagggcga tgccacciac 120
ggcaagctga ccctgaagt catctgcacc accggcaagc tgcccgtgcc ctggcccacc 180
ctcgtgacta ccttcggcta cggcctgatg tgcttcgccc gctaccccgaa ccacatgaag 240
cagcacgact tcttcaagtc cgccatgccc gaaggctacg tccaggagcg caccatcttc 300
ttcaaggacg acggcaacta caagacccgc gccgagggtga agttcgaggg cgacaccctg 360
gttgaaccgca tcgagctgaa gggcatcgac ttcaaggagg acggcaacat cctggggcac 420
aagctggagt acaactacgg tggatccatg catgaccaac tgacagaaga gcagatcgca 480
gaatttaaag aggctttctc cctatttgac aaggacgggg atgggacaat aacaaccaag 540
gagctgggga cggtgatgct gtctctgggg cagaacccca cagaagcaga gctgcaggac 600
atgatcaatg aagttagatgc cgacgtaat ggcacaatcg acttccctga gttcctgaca 660
atgatggcaa gaaaaatgaa agacacagac agtgaagaag aaatttagaga agcgttccgt 720
gtgtttgata aggatggcaa tggctacatc agtgcagcag agcttcgcca cgtgatgaca 780
aaccttggag agaagttAAC agatgaagag gttgtatgaaa tgatcaggga agcagacatc 840
gatggggatg gtcaggtaaa ctacgaagag ttgtacaaa ttagtgcacgc aaaggctac 900
aacagccaca acgtctatat catggccgac aagcagaaga acggcatcaa ggtgaacttc 960
aagatccgcc acaacatcga ggacggcagc gtgcagctcg ccgaccacta ccagcagaac 1020
acccccatcg gcgacggcct cgtgctgctg cccgacaacc actacctgag ctaccagtcc 1080
gccctgagca aagacccaa cgagaagcgc gatcacatgg tcctgctgga gttcgtgacc 1140

gccggccggga tcactatcg catggacgag ctgtacaagt aa 1182

<210> 14

<211> 48

<212> DNA

<213> Artificial Sequence

<220>

<223> BamHI/DEVD F primer

<400> 14

gggggatccg ccatcaagaa tgaaggaaag agaaaaggcg acgaggtg 48

<210> 15

<211> 49

<212> DNA

<213> Artificial Sequence

<220>

<223> NheI/DEVD R primer

<400> 15

ggggctagcg gccacttcat ctgttccatc cacctcgtcg cctttctc 49

<210> 16

<211> 795

<212> DNA

<213> Artificial Sequence

<220>

<223> DEV Dins of Bio-sensor

<400> 16

a~~tt~~ggtagaca agggcgagga gctgtt~~c~~acc ggggtgg~~t~~gc ccatcctggt cgagctggac 60
ggcgacgtaa acggccacaa gttcagcg~~t~~g tccggcgagg gcgagg~~gg~~cg~~a~~ tgccac~~c~~ta~~c~~ 120
ggcaagc~~t~~ga ccctgaagtt catctgc~~a~~cc accggca~~a~~gc tgcccgtgcc ctggcccacc 180
ctcgtgacta ctttcggct~~a~~ cggc~~t~~gat~~t~~ tgcttgc~~cc~~ gctaccccg~~a~~ ccacatgaag 240
cagcacgact tcttcaagtc cgccatgccc gaaggctac~~g~~ tccaggagcg caccatctc 300
ttcaaggac~~g~~ acggcaacta caagacccgc gccgagg~~t~~ga agttcgagg~~gg~~ cgacacccctg 360
gtgaaccgc~~a~~ tcgagctgaa gggcatcgac ttcaaggagg acggcaacat cctggggcac 420
aagctggagt acaactacgg tggatccg~~cc~~ atcaagaat~~g~~ aaggaaagag aaaaggc~~g~~ac 480
gagg~~t~~ggat~~g~~ gaacagat~~g~~a agtggccgct agcaacagcc acaacgtcta ta~~t~~catggcc 540
gacaaggcaga agaacggcat caagg~~t~~gaac ttcaagatcc gccacaacat cgaggacggc 600
agcgtgc~~ag~~c tcgccc~~g~~acca ctaccagc~~ag~~ aacaccccc~~a~~ tcggcgac~~gg~~ cctcgtgct~~g~~ 660
ctgccc~~g~~aca accactac~~ct~~ gagotacc~~ag~~ tccgccc~~t~~ga gcaaagaccc caacgagaag 720
cgcgatcaca tgg~~t~~c~~t~~gct ggagttcg~~t~~g accggccgccc ggatcactct cggcatggac 780
gagctgtaca agt~~aa~~ 795

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR03/00455

A. CLASSIFICATION OF SUBJECT MATTER**IPC7 C07K 14/47**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 : C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korea Patent and Applications for Inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubMed, Delphion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	Nagai T., et al. "Circularly permuted green fluorescent proteins engineered to sense Ca ²⁺ " Proc. Natl. Acad. Sci. USA, 2001, Vol. 98(6): pages 3197-3202, See the abstract.	1-7, 10-11 8-9, 12-15
Y A	Griesbeck O., et al. "Reducing the environmental sensitivity of yellow fluorescent protein" J. Biol. Chem., 2001, Vol. 276: pages 29188-29194, See the abstract.	1-7, 10-11 8-9, 12-15
Y A	Luo K.Q., et al. "Application of the fluorescence resonance energy transfer method for studying the dynamics of caspase-3 activation during UV-induced apoptosis in living HeLa cells" Biochem. Biophys. Res. Commun., 2001, Vol. 283: pages 1054-1060, See the abstract.	1-7, 12-13 8-11, 14-15
Y A	Tyas L., et al. "Rapid caspase-3 activation during apoptosis revealed using fluorescence-resonance energy transfer" EMBO Rep., 2000, Vol. 1(3): pages 266-270, See the abstract.	1-7, 12-13 8-11, 14-15
A	Kim J. E., et al. "Subcellular localization of hepatitis C viral proteins in mammalian cells" Arch. Virol., 1999, Vol 144: pages 329-343, See the abstract.	1-15
A	US 5998204A (Tsien et al.), Dec. 07, 1999, See the whole document.	1-15

 Further documents are listed in the continuation of Box C. See patent family annex.

- * Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

23 JUNE 2003 (23.06.2003)

Date of mailing of the international search report

24 JUNE 2003 (24.06.2003)

Name and mailing address of the ISA/KR

 Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

HAN, Hyung Mee

Telephone No. 82-42-481-5601



INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR03/00455

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5998204 A	Dec. 07, 1999	WO 9840477 A1 AU 6702398 A1 EP 0970199 A1	Sep. 17, 1998 Sep. 29, 1998 Jan. 12, 2000